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# Primary Role of Kupffer Cell-Hepatocyte Communication in the Expression of Oxidative Stress in the Post-Ischaemic Liver

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It has been reported that hepatocyte metabolism and function can be modulated by the activated Kupffer cell through the release of different biomolecules like cytokines, eicosanoids, oxygen free radicals and enzymes. In relation to these paracrine factors involved in circuits of intercellular communication, the existence of a hepatic oxygen sensor located in the Kupffer cell has been postulated. According to this postulate the oxygen metabolism of the liver parenchymal cells could be under the control of the Kupffer cells.

In order to study the role of the Kupffer cell in the reperfusion syndrome of the liver, a lobular ischaemia–reperfusion model was performed in rats with or without previous treatment with gadolinium chloride to block Kupffer cell function. Spontaneous chemiluminescence of the liver surface, oxygen uptake by tissue slices and tert-butyl hydroperoxide-initiated chemiluminescence determinations were performed to evaluate the oxygen metabolism and the oxy-radical generation by the liver. The lower basal photoemission, in parallel with a lower basal oxygen uptake registered in the hepatic lobes from the animals pretreated with gadolinium chloride clearly indicates that the gadolinium chloride-dependent functional inhibition of Kupffer cell leads to a downregulation of oxygen metabolism by the liver. Moreover, the intensity of oxidative stress exhibited by the postischaemic lobes appears to be closely linked with the Kupffer cell activity. On the basis of the data obtained we propose that a paracrine circuit between activated Kupffer cell and hepatocytes is an early key event in the induction of postischaemic oxidative stress in the liver. Furthermore the interference with the mitochondrial electron flow by some biomolecules released from the activated Kupffer cell, such as tumour necrosis factor, interleukins, eicosanoids, etc., would increase the rate of generation of reactive oxygen species by the inhibited mitochondrial respiratory chain. © 1998 John Wiley & Sons, Ltd.

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KEY WORDS — Kupffer cell function; mitochondria; ischaemia–reperfusion; oxidative stress

## INTRODUCTION

The term oxidative stress refers to the pathological condition to which cells are exposed as a consequence of an increased steady-state concentration of reactive oxidant species (ROS).<sup>1</sup> Most of these

molecules are reduced derivatives of molecular oxygen, often with an unpaired electron in the reactive orbital, so chemically defined as free radicals.

Oxidative stress has been proposed as one of the pathogenetic mechanisms of cell damage observed in a variety of organs when a transient period of ischaemia is followed by blood reflow and reperfusion.<sup>2–4</sup> However, little still is known about the molecular mechanisms by which oxidative stress is built up in the postischaemic tissue.

In the particular case of the post-reperfusion damage of the liver, it has been reported that the hepatocytes retain a very high total antioxidant capacity; being in fact able to counteract conditions

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of extremely high oxidative stress without adverse effects.<sup>5</sup> Therefore, it seems unlikely that an increased hepatocyte generation of ROS could significantly and consistently contribute to the hepatic injury that constantly follows reperfusion, as previously suggested.<sup>5,6</sup>

In recent years a crucial role in the pathophysiology of liver reperfusion injury has been attributed to activated Kupffer cells.<sup>7,8</sup> Kupffer cells generate a spectrum of bioactive molecules including eicosanoids, tumour necrosis factor- $\alpha$  (TNF), interleukin-1 (IL-1), interleukin-6 (IL-6), platelet activating factor, hydrolytic enzymes, nitric oxide (NO.) and ROS, in response to soluble and particulate stimuli.<sup>9</sup> To this regard, it has been shown *in vitro* that Kupffer cells are able actively to produce ROS after a cycle of hypoxia–reoxygenation.<sup>10</sup> Therefore, it is conceivable that the activated Kupffer cell is involved in the post-ischaemic damage of the hepatocytes and that such damage is oxidative stress-mediated.

In agreement with this hypothesis, it has been reported that biomolecules released by activated Kupffer cells can operate as paracrine factors to modulate hepatocyte metabolism and function. For example, prostaglandins have been demonstrated to modulate hepatocyte glycogenolysis,<sup>11,12</sup> whereas TNF, IL-1 and IL-6 have been shown to be major stimuli for the hepatic acute-phase response.<sup>13</sup> It has also been reported that two bioproducts normally released by activated Kupffer cell, namely TNF and NO., are able to exert inhibition of the mitochondrial respiratory chain in a mouse fibrosarcoma cell line and in the rat gastrocnemius muscle, respectively.<sup>14,15</sup>

One interesting development concerning non-invasive techniques to estimate oxidative stress *in vivo* is that based on the measurement of increased chemiluminescence by sensitive photon capture techniques applied to an exposed organ in an anaesthetized animal.<sup>16–18</sup> Liver chemiluminescence *in situ* provides an organ-specific and non-invasive method to measure the rate of formation of excited species, mostly singlet oxygen, and represents an indirect but very reliable index of the steady-state level of oxy-radicals and organic-peroxyradicals.<sup>16–18</sup>

Liver chemiluminescence has been adopted to monitor oxidative stress in the experimental *in vivo* model designed to evaluate the role of Kupffer cells in the ischaemia–reperfusion syndrome. Rats were subjected to a selective hepatic ischaemia–reperfusion cycle, a subgroup being pretreated with

gadolinium chloride (GdCl<sub>3</sub>), a specific blocker of Kupffer cell function.<sup>19</sup>

Evidence is provided of intercellular communication between Kupffer and parenchymal cells in the generation of oxidative stress in the post-ischaemic rat liver. In addition, a general role for the Kupffer cell in the regulation of oxygen consumption by the liver is here proposed.

## MATERIALS AND METHODS

### *Animals*

Male Wistar rats weighing 180–200 g fed a conventional laboratory diet and water *ad libitum* were maintained under alternating 12 h cycles of light and dark until the start of the experiment. All animals received human care in compliance with the institutional guidelines.

### *Experimental Model*

The animals were heparinized (440 IU kg<sup>-1</sup> body weight, i.p.) and then anaesthetized with sodium pentobarbital (50 mg kg<sup>-1</sup> body weight, i.p.) diluted in a sterile 0.9 per cent NaCl solution (w/v). The liver was exposed by a midline abdominal incision. The liver lobes were gently moved to expose the hilum and then the right branches of the hepatic artery and portal vein were occluded with an atraumatic bulldog clamp (Roboz Surgical Instrument Co., Washington, DC), rendering about one-third of the total hepatic mass ischaemic. As the blood flow from the portal vein continued to pass through the medial, the left lateral and the caudate lobes splanchnic congestion was prevented. According to previous results,<sup>4</sup> the increased blood supply to these lobes did not produce any modification of their functions, as indicated in terms of oxygen uptake and surface photon emission. By this view it is possible to assume that the medial, left lateral and caudate lobes represent control tissue. Then the abdominal cavity was closed and the rats were positioned under warming lamps to maintain a constant body temperature. After 3 h of ischaemia, reperfusion was initiated by removal of the clamp. The animals received 2 ml of a sterile 0.9 per cent NaCl solution (w.v) subcutaneously and the wound was closed with 3–0 silk. The rats were allowed to recover and after 0.5 h of reperfusion the animals were

reanaesthetized and the abdominal cavity was opened again.

#### *Treatment*

Kupffer cell activity was inhibited by the administration of  $\text{GdCl}_3$ , (Aldrich Chemical Co., Milwaukee, WI), ( $10 \text{ mg kg}^{-1}$  body weight) diluted in a sterile 0.9 per cent NaCl solution (w/v) and administered through the tail vein 24 h before the start of the ischaemia–reperfusion cycle. Animals without treatment used as a control group, received an equivalent volume of a sterile 0.9 per cent NaCl solution (w.v).

#### *Spontaneous Liver Surface Chemiluminescence*

Spontaneous liver surface chemiluminescence of the right lateral lobes of the *in situ* liver was monitored during the ischaemia–reperfusion cycle using a photon counter (Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA) with a model 9658 photomultiplier (responsive in the range 300–900 nm; Thorn EMI, Ruislip, Middlesex, U.K.) as described.<sup>16–18</sup> The emission was expressed as counts per second per square centimetre of liver surface (c.p.s.  $\text{cm}^{-2}$ ).

#### *Sample Heading*

At the end of the reperfusion phase the animals were killed and fragments from the postischaemic and the control lobes were taken for processing.

#### *Oxygen Uptake*

Liver slices (1 mm thick) from the postischaemic and the control lobes were placed in Krebs–Ringer solution containing 10 mM glucose in the reaction chamber of a Clark-type oxygen electrode at  $30^\circ\text{C}$ . The medium was air equilibrated at 0.22 mm oxygen. The initial rate of oxygen uptake was monitored during 10 min. The results were expressed as  $\mu\text{mol oxygen min}^{-1} \text{ g}^{-1}$  of tissue.<sup>20</sup>

#### *Liver Homogenates*

Liver homogenates were prepared in a medium consisting of 120 mM KCl and 30 mM phosphate buffer (pH 7.4) and centrifuged at 600 g for 10 min at  $4^\circ\text{C}$  to remove nuclei and cellular debris. The supernatants were used as ‘homogenates’.

#### *Tert-Butyl Hydroperoxide-Initiated Chemiluminescence*

Tert-butyl hydroperoxide (T-BOOH)-initiated chemiluminescence was measured in a liquid scintillation counter in the ‘out-of coincidence’ mode.<sup>21</sup> Homogenates were placed in low photoemission glass vials in a reaction medium consisting of 120 mM KCl, 30 mM phosphate buffer (pH 7.4). Measurements were started by the addition of 3 mM tert-butyl hydroperoxide. The background level of emission of the empty vials was 2500–3000 counts per min (c.p.m.). Determinations were carried out at  $30^\circ\text{C}$  with occasional stirring. The results, registered in c.p.m.  $\text{mg}^{-1}$  of protein, were expressed as the ratio between the initiated chemiluminescence of the lobes subjected to ischaemia–reperfusion cycles (designated B) and the control lobes (designated A). In the basal condition, previous to the start of the ischaemia–reperfusion cycles, the ratio between the lobes was  $1.0 \pm 0.1$ .

#### *Protein Determination*

Protein was measured by the method of Lowry *et al.*<sup>22</sup> using bovine serum albumin as standard.

#### *Statistical Analysis*

All the numbers in the tables and figures represent mean values of three experiments  $\pm$  standard error of the mean (SEM). The differences between both experimental groups were analysed statistically by the Student’s *t*-test for unpaired samples.<sup>23</sup>

## RESULTS

#### *Spontaneous Liver Surface Chemiluminescence*

Exposure of liver to a sensitive phototube allows the direct detection of spontaneous chemiluminescence of the *in situ* organ under physiological conditions. The surface chemiluminescence of the liver *in situ* at time zero of the experiment, i.e. previous to the start of the ischaemia–reperfusion cycles, was  $13 \pm 2$  and  $7 \pm 1$  c.p.s.  $\text{cm}^{-2}$  for the non-treated and the  $\text{GdCl}_3$ -treated groups respectively. The photon emission by the livers of the  $\text{GdCl}_3$ -treated rats indicates a lower singlet oxygen concentration with respect to the physiological steady state level of singlet oxygen, estimated to be about  $10^{-14} \text{ M}$ .<sup>16–18</sup> The light

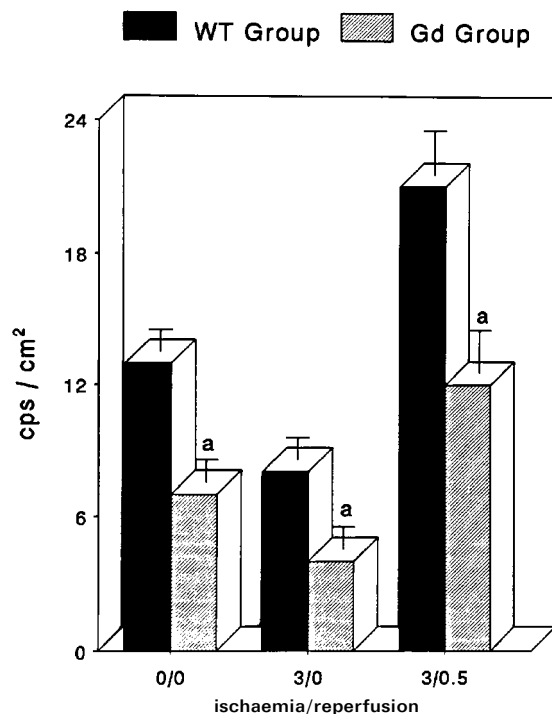


Figure 1. Spontaneous chemiluminescence of the liver surface during a cycle of lobular ischaemia-reperfusion. 0/0, Spontaneous chemiluminescence previous to the start of the cycle of ischaemia-reperfusion; 3/0, spontaneous chemiluminescence of the lobes subjected to 3 h of ischaemia; 3/0.5, spontaneous chemiluminescence of the lobes subjected to 3 h of ischaemia and 0.5 h of reperfusion. WT, animals without treatment; Gd, animals treated with  $\text{GdCl}_3$  (see Materials and Methods). a, significantly different with respect to the WT group ( $p < 0.01$ ).

emission detected in the ischaemic lobes from both experimental groups just before declamping was 1/3 lower than the relative values registered at time zero, indicating a low ROS generation due to the fall in the availability of molecular oxygen. When the blood flow was restored, the chemiluminescence emission by the postischaemic lobes from both treated and  $\text{GdCl}_3$ -treated rats showed a rapid and strong increase. Once again, the percentage variation was similar in the two experimental groups, but overall, spontaneous chemiluminescence in the reperfused liver was significantly higher in non-treated rats ( $21 \pm 2$  versus  $12 \pm 2$  c.p.s.  $\text{cm}^{-2}$ ;  $p < 0.01$ ) (Figure 1).

#### Oxygen Uptake

The treatment with  $\text{GdCl}_3$  altered the oxygen uptake by the liver. In fact, tissue slices from the

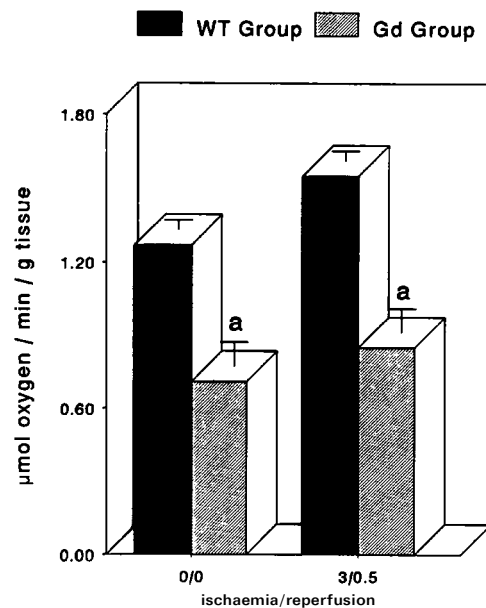


Figure 2. Oxygen uptake by liver slices after a cycle of lobular ischaemia-reperfusion. 0/0, Rate of oxygen uptake of the lobes previous to the start of the cycle of ischaemia-reperfusion; 3/0.5, rate of oxygen uptake of the lobes subjected to 3 h of ischaemia and 0.5 h of reperfusion. WT, animals without treatment; Gd, animals treated with  $\text{GdCl}_3$  (see Materials and Methods). a, significantly different with respect to the WT group ( $p < 0.01$ ).

control lobes of non-treated rats showed a rate of oxygen uptake of  $1.27 \pm 0.04 \mu\text{mol min}^{-1} \text{g}^{-1}$  of tissue liver, whereas a 44 per cent decrease in the rate of oxygen consumption was detected in the control lobes from the  $\text{GdCl}_3$  group. An increased rate of oxygen uptake was evident after reperfusion in the non-treated group, indicating an additional consumption of oxygen due to oxy-radical generation and lipid peroxidation. A lower rate of oxygen uptake was detectable in the tissue slices from the postischaemic lobes of  $\text{GdCl}_3$ -treated rats, that were not statistically different from its relative basal control (Figure 2).

#### *Tert-Butyl Hydroperoxide-Initiated Chemiluminescence*

Tissue homogenates from postischaemic and control lobes of the non-treated and the  $\text{GdCl}_3$ -treated rats were subjected to *in vitro* oxidative stress by incubation in the presence of T-BOOH. This assay allows determination of the integral level of endogenous chain-breaking antioxidants and, indirectly, the previous occurrence of oxidative

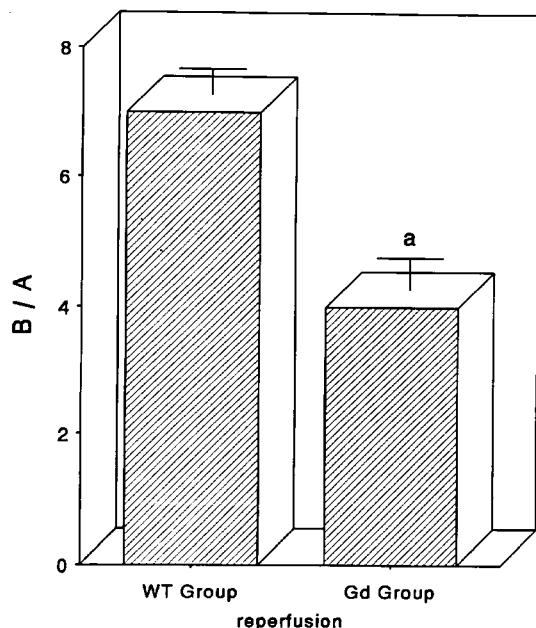


Figure 3. Tert-butyl hydroperoxide-initiated chemiluminescence of the liver after a cycle of lobular ischaemia–reperfusion. The measurement was done 0.5 h after reperfusion of the lobes previously subjected to 3 h of ischaemia. The results are expressed as the ratio between the initiated chemiluminescence of the postischaemic lobes (designated B) and their respective control lobes (designated A). WT, animals without treatment; Gd, animals treated with  $\text{GdCl}_3$  (see Materials and Methods). a, significantly different with respect to the WT group ( $p < 0.01$ ).

stress in a tissue with marked tissue damage.<sup>21</sup> As shown in Figure 3, the T-BOOH-initiated chemiluminescence of the homogenates from the lobes subjected previously to ischaemia–reperfusion of the non-treated and the  $\text{GdCl}_3$ -treated animals was higher than the photoemission recovered in their respective internal controls, with values of the B/A ratio greater than  $1.0 \pm 0.1$ . The difference in T-BOOH initiated chemiluminescence exhibited by the two experimental groups after reperfusion was statistically significant ( $p < 0.01$ ).

## DISCUSSION

Because the liver is well suited for antioxidant capacity, the physiological steady-state concentration for superoxide anion and hydrogen peroxide is maintained in the order of  $10^{-11}$  M and  $10^{-7}$  M, respectively.<sup>24,25</sup> However, under experimental pathological conditions, e.g. ischaemia reperfusion,<sup>4</sup> suprahepatic vein occlusion,<sup>26</sup> acetaminophen toxicity,<sup>27</sup> ethanol toxicity,<sup>28</sup> vitamin

E-selenium deficient diets<sup>29</sup> and mitoxantrone and doxorubicin toxicity,<sup>30</sup> a marked change occurs in the equilibrium between prooxidant and antioxidant reactions, leading to reported biochemical and histological signs of liver damage.

Under physiological conditions, mitochondria account for about 15 per cent of the hepatocytes ROS generation.<sup>31</sup> Two sites have been described in the mitochondrial respiratory chain related to the superoxide anion production: NADH-ubiquinone oxidoreductase (complex I) and ubiquinone cytochrome c reductase (complex III). The amount of anion superoxide production by the mitochondrial respiratory chain at complexes I and III normally occurs at a rate of  $0.6 \text{ nmol min}^{-1} \text{ mg}^{-1}$  of protein and  $3.2 \text{ nmol min}^{-1} \text{ mg}^{-1}$  of protein, respectively.<sup>32,33</sup> However, the extent of the intramitochondrial anion superoxide and hydrogen peroxide production is increased up to maximal values when the respiratory chain is under fully reduced conditions, such as when mitochondrial inhibitors of the electron transport are used.<sup>34,35</sup> During ischaemia, the lack of the electron acceptor, molecular oxygen, and the inhibition of the electron transport system by ischaemia by-products and/or morphological changes, could lead to a high level of reduction of the components of the mitochondrial respiratory chain. Upon reperfusion, a burst of anion superoxide would be expected to occur owing to the increased autooxidation rate of the intramitochondrial sources of the anion superoxide, namely the semiquinone ( $\text{QH}^\cdot$ ) of complexes I and III.<sup>4</sup>

Over the last few years, a likely regulation of hepatic function by the interaction of specific cell populations in the liver has become increasingly evident. The bioactive molecules generated by activated Kupffer cells in response to a variety of stimuli have been shown to modify the hepatocyte function, as mentioned before.<sup>11–13</sup> In addition, Kupffer cells appear to be implicated in the regulation of the hepatic oxygen consumption. Actually, it has been postulated that in response to oxygen stimulus, the Kupffer cell produces specific signal molecules (eicosanoids and/or cytokines) that regulate the oxygen uptake by liver paranchymal cells.<sup>36</sup> In a low flow–reflow model of rat liver perfusion, the authors obtained the following findings: (1) activation of Kupffer cells was associated with an increase in the oxygen uptake by the liver; (2) oxygen uptake during reflow, when Kupffer cells were activated, was significantly greater than the basal values; (3) oxygen uptake

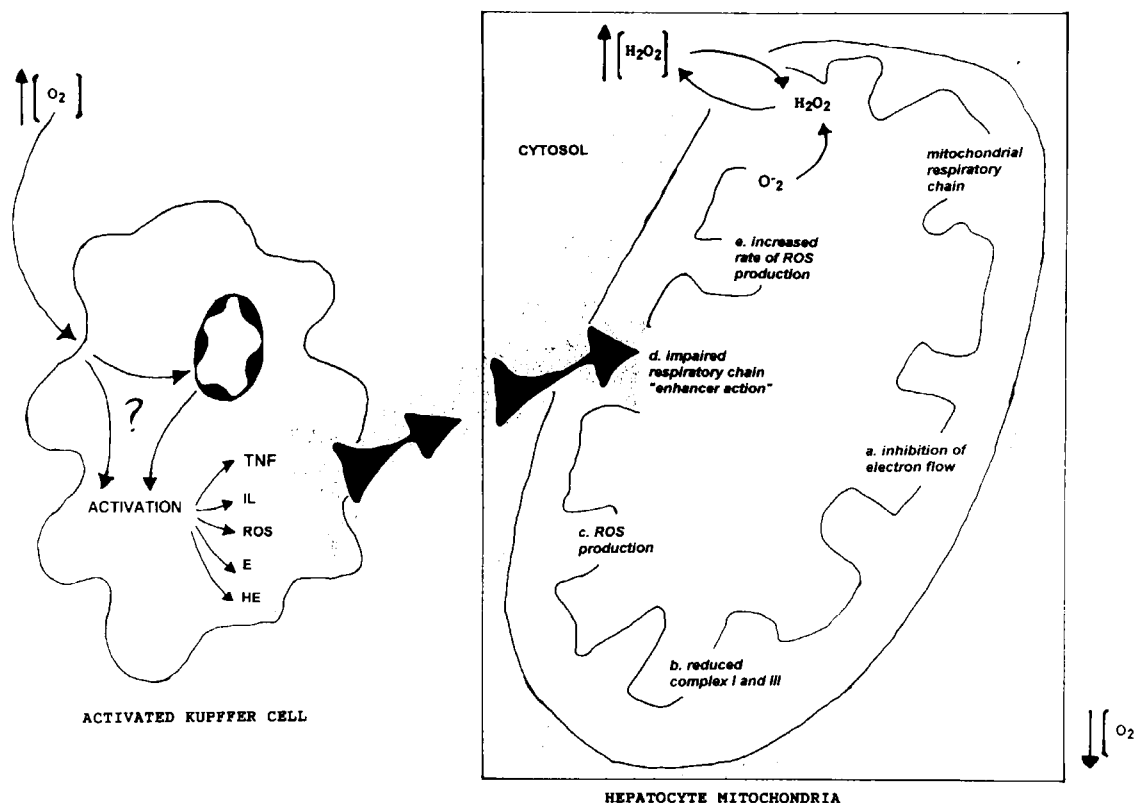


Figure 4. Proposed mechanism to explain the ROS production by hepatocyte mitochondria associated with ischaemia-reperfusion as the result of the Kupffer cell-hepatocyte communication.  $O_2$ , molecular oxygen;  $O_2^-$ , anion superoxide;  $H_2O_2$ , hydrogen peroxide; ROS, reactive oxygen species; TNF, tumour necrosis factor; IL, interleukins; E, eicosanoids; HE, hydrolytic enzymes.

did not increase over basal values at reflow in liver of rats previously treated with methyl palmitate, an inhibitor of Kupffer cell function. Taken together these data allowed the authors to suppose the existence of a hepatic oxygen sensor located in the Kupffer cell.<sup>36</sup>

In order to examine the participation of the Kupffer cell in a model of ischaemia-reperfusion of the liver, we used a specific inhibitor of its function, namely  $GdCl_3$ . The hepatic lobes from the animals pretreated with  $GdCl_3$  showed, with respect to those from the animals used as control group, a lower basal photonemission, corresponding to a lower basal oxygen uptake. These results clearly indicate that the  $GdCl_3$ -dependent functional inhibition of Kupffer cells leads to a down-regulation of oxygen metabolism by the liver. A significant difference in spontaneous chemiluminescence between the two experimental groups was also maintained during both ischaemia and reperfusion. Hence, the intensity of oxidative stress appears to be tightly

dependent on Kupffer cell activity. This concept is strengthened by the consistent finding achieved when T-BOOH-initiated chemiluminescence was measured *ex vivo* in both groups after a cycle of ischaemia-reperfusion.

A few years ago, our group proposed that the increased ROS production by inhibited mitochondria, owing to the increased autooxidation rate of the major intramitochondrial sources of anion superoxide, appeared to be the initial cause of oxidative stress during the early reperfusion in the rat liver.<sup>4</sup> New experimental evidence now points to the direct participation of TNF on the generation of superoxide anion by the mitochondrial respiratory chain as a possible mechanism of TNF-induced cytotoxicity. In fact, it has been reported that TNF-induced inhibition of mitochondrial electron transport was able to damage the mitochondrial chain at complex III with an increased production of ROS inside the mitochondria.<sup>14, 37</sup>

Since the Kupffer cell is one of the most important sources of TNF production,<sup>13</sup> it seems reasonable to expect an increased TNF release once Kupffer cells are activated by an ischaemia–anoxia/reperfusion–reoxygenation cycle.<sup>7, 8, 10</sup> In support of this view, a transient elevation of TNF, as well as of IL-1 plasma concentrations, was observed after a reperfusion phase in a total ischaemia rat liver model.<sup>38</sup> After being internalized by neighbouring hepatocytes, TNF and maybe other Kupffer cell bioproducts would interfere with the mitochondrial electron flow, most probably at complex III, increasing the rate of ROS production by the inhibited mitochondrial respiratory chain. In this sense, TNF could act as an enhancer for the generation of ROS by the reduced components of the mitochondrial respiratory chain.

On the other hand, when Kupffer cell function is inhibited by GdCl<sub>3</sub>, less intensive oxidative stress is actually detectable in the reperfused hepatic lobes, possibly due to the lack of enhancers like TNF.

On the basis of these data Figure 4 summarizes the proposed communication between the activated Kupffer cell and the hepatocyte as an early key event in the induction of postischaemic oxidative stress of the liver.

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